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㉖ **Secretory signal selection vectors for extracellular protein synthesis in bacilli.**

㉗ Novel vectors are provided for identifying secretory signal sequences from DNA fragments of unicellular microorganisms. The plasmids comprise a multiple cloning site with restriction sites in reading frame with a structural gene which permits rapid screening of the secreted expression product. Optionally, the vectors may include a promoter region upstream from the multiple cloning site. The invention is exemplified with Bacillus. Specific secretory signal sequences have been isolated with those vectors, allowing real secretion of proteins in economically high yields. Certain isolated signal sequences appeared to be much better than the sequences regulating the high yield secretion of known extracellular proteins.

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SECRETORY SIGNAL SELECTION VECTORS FOR EXTRACELLULAR PROTEIN SYNTHESIS IN BACILLI

FIELD OF THE INVENTION

This invention relates to secretory signal selection vectors and the use of the secretory signal sequences picked up with these vectors for high yield production of extracellular proteins, preferably enzymes, in recombinant DNA microorganisms of the genus Bacillus.

BACKGROUND OF THE INVENTION

Bacilli have a very long history of use in industrial large scale fermentation because of some very advantageous properties. They are capable of growing on rather cheap nutrients, they do not produce endotoxins so that they are harmless for men, plants and animals, and they are capable of synthesizing many industrially interesting proteins, such as enzymes, as extracellular products, which in most cases can be isolated rather easily. This combination of characteristics, in particular the secretion of products into the culture medium, makes the use of Bacilli for commercial purposes much more attractive than for example the use of E. coli. In order to improve the economic value of Bacilli even further, many attempts have been made to develop a Bacillus host-vector system, both for homologous and heterologous gene expression. See, for example, D. Dubnau in "Experimental Manipulation of Gene Expression", Academic Press (1983) 33-51 and R.H. Doi, Biotechnology and Genetic Engineering 2 (1984) 126-155.

Unfortunately, however, the level of expression and secretion appeared to be relatively low. See, for example, S. Kovacevic et al., J. Bacteriol. 162 (1985) 521-528, C.W. Saunders et al., J. Bacteriol. 157 (1984) 718-726, R. Ohmura et al., J. Biochem. 95 (1984) 87-93, Book of Abstracts of the 3rd International Conference on Genetics and Biotechnology of Bacilli, Stanford, USA (1984), K. Lundström, FEMS Letters 23 (1984) 65-70, I. Palva et al., Gene 22 (1983) 229-235, K. Lundström et al., Virus Res. 2 (1985) 69-83, K. Hardy et al., Nature 293 (1981) 481-483, K. Mosbach et al., Nature 302 (1983) 543-545, S. Chang et al., NSC Ser. 4 (1982) 254-261, D.M. Williams et al., Gene 16 (1981) 199-206 and J.I. Flock et al., Mol. Gen. Genet. 195 (1984) 246-251.

Many improvements of the Bacillus cloning system have already been reported with respect to increased expression to an economically acceptable level. They are mainly related to improvements of the promoter region in order to increase the transcription efficiency (D.M. Williams et al., J. Bacteriol. 146(1981) 1162-1165, R.G. Schoner et al., Gene 22 (1983) 47-57, D.S. Goldfarb et al., Nature 293 (1981) 309-311, L. Band et al., Gene 26 (1983) 313-315, C.E. Donnelly and A.L. Sonnenschein, J. Bacteriol. 157 (1984) 965-967 and European Patent Application No. 86201951.0), but they also concern improvements of the Shine-Dalgarno region in order to increase the translation efficiency (A. Hui et al., EMBO Journal 3 (1984) 623-629, H. de Boer et al., DNA 2 (1983) 231-235, L. Band and J. Henner, Biochem. Soc. Symp. 48 (1984) 233-245 and European Patent Application No. 86101951.0). The construction and use of vectors that permit isolation, improvements and use of secretory signal sequences, however, have not been reported so far.

Most of the current understanding of protein secretion in prokaryotic cells is limited to E. coli (L.L. Randall and S.J.S. Hardy, Microbiol. Reviews 48 (1984) 290-298 and S.A. Benson et al., Ann. Rev. Biochem. 54 (1985) 101-134). In E. coli, a gram-negative organism, the secretion of a protein is in fact the transport to the periplasm rather than to the culture medium as is the case in Bacillus, a gram-positive organism. The knowledge of protein secretion in E. coli has mainly been obtained by the study of fusion proteins containing a segment of a protein which is known to be translocated to the periplasm, attached to beta-galactosidase.

A very serious limitation in the use of such hybrid proteins is that the beta-galactosidase moiety appears to be unable to pass through the E. coli cytoplasmic membrane, cf. C.S. Hoffman and A. Wright, Proc. Natl. Acad. Sci. USA 82 (1985) 5107-5111. Although this problem has been overcome by the use of alkaline phosphate instead of beta-galactosidase (C. Monoil and J. Beckwith, Proc. Natl. Acad. Sci. USA 82 (1985) 8129-8133), the authors describe their results, obtained with some hybrid alkaline phosphatases as transport from the cytoplasm into the periplasm rather than as secretion into the culture medium.

SUMMARY OF THE INVENTION

This invention provides for secretory signal sequence selection vectors which allow for isolation of signal sequences providing secretion of proteins in economically high yields. It was surprisingly found that certain signal sequences are much more efficient than other sequences regulating high secretion levels of known extracellular Bacillus proteins. Therefore, this invention provides an improvement in extracellular protein yields, both of proteins, homologous and heterologous, which were not secreted efficiently so far and of proteins (for example, enzymes) that are already secreted efficiently.

DESCRIPTION OF THE FIGURES

Fig. 1 - Construction of plasmids pGPA11, pGPB11 and pGPP11

This figure shows the cloning strategy for the constructions of pGPA11, pGPB11 and pGPP11. The origins of plasmid segments are indicated in the key. Only those restriction sites relevant for the construction and properties of the recombinant plasmids are shown.

Fig. 2 - Construction of plasmid pHP12

This figure shows the cloning strategy for the construction of pHP12. The origins of plasmid segments are indicated in the key. Only those sites relevant for the construction and properties of the recombinant plasmid are shown. Plasmid pHP14 was constructed similarly, except that the 1.6 kb Aat II-AhaIII fragment was taken from pUC13.

Fig. 3 - Construction of plasmids pGPA14, pGPB14 and pGPP14

This figure shows the DNA sequences at the fusion points of the multiple cloning sites (MCS) with the structural genes (A: alpha-amylase; B: beta-lactamase; C: penP). The vertical arrows in Fig. 3A and 3C indicate the original cleavage sites for leader peptidase. Several codons around the processing sites are numbered and the corresponding amino acids are indicated in italics. The plasmids pGPA14, pGPB14 and pGPP14 derivatives were constructed by digesting pGPA11, pGPB11 and pGPP11, respectively, with PstI followed by chewing back for blunt ending with T4 DNA polymerase. In Fig. 3C, L indicates the processing site to the large extracellular form of penicillinase; M indicates the membrane-bound form of penicillinase. The plasmids pGPA14, pGPB14 and pGPP14 were deposited in Bacillus subtilis strain on May 2, 1986 under the provisions of the Budapest Treaty with the Centraal Bureau of Schimmelcultures, Oosterstraat 1, NL-3742 SK Baarn, and were allotted numbers CBS 245.86, CBS 246.86 and CBS 247.86, respectively.

Fig. 4 - Construction of plasmids pGPB15 and pGPB16

This figure shows schematically the construction of alpha-amylase-beta-lactamase and penP-beta-lactamase gene fusions. Several codons around the PstI sites are numbered. The line ---- indicates a fragment derived from pGK13-alpha-amylase; the line -.-.-a fragment derived from pRW101, the line _____ a beta-lactamase gene from pGPB11 and the line + + + + + charged amino acid residues. For details, see Example V.

Fig. 5 - Construction of plasmids pGPP3 and pGPP5

This figure shows the penP-penP and the alpha-amylase-penP gene fusions. In pGPP3 the original penP signal sequence encoding region is restored, whereas in pGPP5 a hybrid signal sequence region is constructed. For details, see Example V. The part indicated as -.-.- is derived from pRW101, and the part indicated as ---- from pGK13-alpha-amylase.

Fig. 6 - Amounts of beta-lactamase in the culture supernatant of 8G-5 (pSPB8)

The recombinant bacteria (cf. Table 2) were grown in TY and in minimal medium. Beta-lactamase activities were determined with nitrocefin as described hereinafter on pp. 16-17. The growth of the bacteria was monitored by the absorbance at 450 nm. ●-●- ; A_{450}/TY ; ▲-▲-▲- ; $A_{450}/\text{minimal medium}$; ○-○-○- , beta-lactamase activity in TY medium; △-△-△- , beta-lactamase activity in minimal medium.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for detecting signal sequences involved with secretion in unicellular microorganisms, particularly prokaryotic organisms. The methods are employed for identifying signal sequences present in the host genome, on the genome of other organisms and on other DNA fragments (identified or unidentified), evaluating such sequences, and then being able to isolate the sequences and use them with other than the wild-type gene to provide for efficient secretion of polypeptide sequences of interest, more particularly sequences foreign to the expression host. By employing the subject invention, the signal sequence can be detected and be evaluated with a plurality of genes other than the wild-type gene associated with the signal sequence. Efficient signal sequences discovered in this manner may then be utilized for the efficient secretion of products of interest, such as mammalian proteins, enzymes, both prokaryotic and eukaryotic, biocides, structural polymers, or the like.

The subject invention is exemplified by a determination of signal sequences in *Bacillus subtilis* and *Escherichia coli*. While *E. coli* is found to be a particularly useful cloning host, and would be the host of interest for the cloning of DNA regardless of the source of the signal sequences, *B. subtilis* may be considered exemplary of the *Bacillus* organisms, such as *B. thuringiensis*, *B. stearothermophilus*, *B. licheniformis*, as well as other prokaryotic organisms.

The method employs using vectors which serve for cloning and screening of genomic or cDNA for the presence of signal sequences, usually of unidentified fragments. The vectors are characterized by having a plurality of functional sequences. The vectors have at least one, usually two origins of replication, one for the screening/expression host, which is preferably a *Bacillus* host, and the other for a cloning host, which is preferably *E. coli*. The latter origin of replication is usually inserted for convenience only. In a specific embodiment of the invention the cloning and expression hosts are the same, preferably a *Bacillus* host, so the vectors may comprise only one origin of replication. Usually, for the genomic screening, the replication system will be of a relatively low copy number, usually at least 1 and not more than about 500, more usually not more than about 200 copy numbers. The copy number in the cloning host is primarily one of convenience, it is not of significance to this invention.

The vector optionally has 1 or more promoters, usually 0 to 1 promoter which is functional in the screening host. The presence of the promoter allows for detection of signal sequences which have been separated from their wild-type promoter, and for the detection of new signal sequences, namely coding for sequences which may act as secretory signal sequences but normally do not possess this activity. In this manner, relatively small fragments may be screened and the presence of the signal sequences detected. Furthermore, the efficiency of the signal sequence is providing for efficient production of the desired peptide will be separate from the efficiency of the promoter. However, since one can determine the amount of cytoplasmic peptide as compared to secreted peptide, this ratio can be used to evaluate the efficiency of the signal sequence for secretion.

The next functional element in the vector, which may be the first functional element in the absence of the promoter, is a multiple cloning site (MCS). The MCS is characterized by having at least 2, usually at least 3, generally not more than 10, preferably not more than 6, restriction sites, at least 2 of which, and preferably all, are unique to the vector. That is, the vector will be capable of being cleaved uniquely in the MCS. The cloning sites may be blunt ended or have overhangs of from 1 to 4-nucleotides, preferably having overhangs. Desirably, the overhangs will be capable of both, hybridizing with the overhangs obtained with restriction enzymes other than the restriction enzyme which cleaves at the restriction site in the MCS, and hybridizing with the overhangs obtained with the same restriction enzyme. While insertion of a fragment derived by restriction with a restriction endonuclease other than the restriction endonuclease employed for cleaving the vector will result in the inability to excise the insert, nevertheless, in some situations this may be desirable. In some instances this may allow for the use of small fragments by using a plurality of restriction endonucleases having different recognition sites or by using restriction endonucleases which recognize four instead of six nucleotides.



The MCS will usually be not more than about 100 nucleotides, usually not more than about 60 nucleotides, and generally at least about 6 nucleotides, more usually at least about 10 nucleotides.

Desirably, the MCS will be free of stop codons in the translational reading frame for the structural genes. Where a convenient MCS is commercially available, the MCS may be modified by cleavage at a restriction site in the MCS and removal or addition of a number of nucleotides other than 3 or a multiple of 3, which results in a change in reading frame. In this manner, the reading frame will be changed so as to avoid termination of expression at a site internal to the MCS. Desirably, the MCS will be relatively free of codons which might interfere with the efficient secretion of the expression product. To that extent, at least 50% of the codons coded for by the MCS will code for neutral amino acids, desirably at least about 80%. Preferably, at least about 60% of the codons will be aliphatic, where the side chain may be polar, e.g. having an oxy, thio or amido functionality or non-polar, consisting solely of hydrogen and carbon. In addition, the MCS will usually be free of a processing signal.

In referring to a signal sequence, the signal sequence will normally have three regions. The first region will normally be a hydrophilic region, usually including one, more usually including at least about two positively charged amino acids, e.g. K or R. The charged region will usually be followed by a hydrophobic region, where the majority of the amino acids will be non-polar amino acids. The hydrophobic region will then be followed by a more polar region harbouring the processing signal, which may involve two or more amino acids, which are recognized by a particular leader peptide enzyme which cleaves at the processing site. Processing signals may involve as many as eight amino acids or more and may be influenced by the amino acids present immediately adjacent to the processing site in the mature peptide.

The MCS may provide a chain of two or more amino acids between the genomic fragment and the expression product. Usually, the MCS will provide fewer than 30 amino acids, preferably fewer than about 20 amino acids. Of course, the number of amino acids introduced by the MCS will depend not only upon the size of the MCS, but also the site at which the genomic fragment is inserted into the MCS.

Joined to the MCS will be the expression product. The expression product may or may not include the C-terminal portion of a signal sequence or the wild-type processing signal associated with the expression product. Desirably, a plurality of expression products are employed in different vectors, where one vector may employ a gene having a processing signal and the other vector may employ a gene lacking a processing signal. In this manner, by comparing the resulting products, one can determine whether the fragment does or does not include an efficient processing signal.

The gene which is employed will have a number of constraints. One constraint is that the gene can be detected readily, either by detection of a metabolite, resistance to a biocide, e.g. antibiotic, or other means which allows for rapid screening of secretion of the expression product. For the most part, the expression product will be an enzyme, which produces a readily detectable metabolite, e.g. amylase, either alpha- or beta-amylase; which provides for protection from an antibiotic, e.g. beta-lactamase, penicillinase, or bacteriocin. While it is feasible to screen the supernatant using various assays, e.g. immunoassays, Western blots, or the like, for detection of the expression product in the supernatant, preferably the expression product should allow for rapid screening and only after detection of positive clones will it usually be desirable to quantitate the amount of expression product in the supernatant. In addition, it will usually be of interest to determine the amount of cytoplasmic expression product. Since the cytoplasmic product will, for the most part, be unprocessed, it is desirable that the expression product be active, both with and without the N-terminal leader.

Finally, the vector may also include one or more markers. By marker is intended a gene capable of expression in the particular host which allows for ease of selection of those hosts containing the vector. The marker may provide resistance to a biocide, e.g. antibiotic, as described above, as well as other antibiotics, such as erythromycin, actinomycin, chloramphenicol, tetracycline, etc., complement auxotrophic hosts to provide prototrophy, or the like. For each of the hosts involved, where the cloning and screening hosts require different markers, there will usually be two markers present. In this way, the clones may be rapidly screened to distinguish those clones which contain the vector and those clones which do not.

The genomic screening may be done with either a genomic library, that is a restriction endonuclease or sheared product from the chromosome and any extrachromosomal elements present in the host, or a cDNA library, where care must be taken to ensure that the sequence coding the N-terminal sequence has been obtained during reverse transcription and replication. The fragments which are employed will usually be at least about 45 bp, not more than about 5 kbp, usually not more than about 2 kbp and preferably at least about 60 bp. Since the fragments are random, they may not only include the promoter and non-coding 5'

sequence, but also the sequence encoding the signal sequence and processing signal and even portions of the mature expression product. The restricted fragments will be obtained with at least one restriction endonuclease, frequently a plurality of restriction endonucleases, where the restriction endonucleases may provide for the same or different overhangs or for blunt ended fragments.

5 A number of different techniques can be employed to control the size of the fragment. For example, one can use a restriction endonuclease providing a complementary overhang and a second restriction endonuclease to recognize a relatively common site, but provides a terminus which is not complementary to the terminus of the vector restriction site. After joining the fragments to the cleaved vector, one may further subject the resulting linear DNA to additional restriction enzymes, where the vector lacks recognition
10 sites for such restriction enzymes. In this way, a variety of sizes can be obtained.

Where the vector provides a promoter, one can afford to use smaller fragments than when the vector does not provide the promoter. Thus, it will be desirable in many instances to either employ larger fragments or to have a promoter present in the vector which is upstream from the MCS and allows for transcription of the insert in conjunction with the expression product.

15 After the insert has been inserted into the vector and manipulated in any convenient manner, the vector may then be transformed into the cloning host. It may be desirable to provide for expression, as well, in the cloning host. In this instance, either the promoters of the genomic source will be functional in the cloning vector, or a promoter will be provided which provides for transcription of the expression construct in the cloning host. In this manner, one may in addition determine whether the signal sequence and processing
20 signal is recognized by the cloning host, as well as the genomic source host. Thus, one can obtain some evidence of the presence of a signal sequence, since a positive result will be highly indicative of the presence of a signal sequence functional in the expression host, although not dispositive. Sometimes it may be desirable to clone and screen in the expression host. In such case the vector plus insert may be transformed directly into the expression host.

25 Any technique may be used for introduction of the vector into the cloning host, as well as the expression host. Various techniques include conjugation, transformation, transfection, electroporation, fusion, or the like. The resulting transformants may then be grown on an appropriate nutrient medium providing for selection of those cloning hosts containing the vectors. The vectors may then be isolated and screened by transformation into an appropriate expression host and grown in a nutrient medium appropriate to the host.
30 Efficient secretion may be determined by screening the supernatants for the presence of the secreted product. Thus, depending upon the nature of the expression product, various reagents may be added during or after growth which, for example, produce a detectable signal, e.g. a colored reaction, are lethal to the host in the absence of the secreted expression product, or the like. In this manner, one can rapidly screen a large number of clones for efficient secretion.

35 Those clones which appear to be promising may then be further analyzed in a variety of ways. The insert may be excised, using the flanking restriction sites, either those employed for insertion or those present in the MCS which were not employed and isolating the resulting fragment. This fragment may then be sequenced, so that the initiation codon and signal sequence may be determined, based on the coding characteristics. The protein product may be sequenced to determine the site at which processing occurred.
40 The nucleic acid sequence may then be used as a probe to determine the wild-type gene which employs the particular signal sequence. The signal sequence may be isolated or it may be desirable to isolate a DNA sequence encoding the signal sequence and processing signal in conjunction with a few codons of the wild-type gene downstream from the processing signal. The extra codons may enhance secretion efficiency. In addition, one may obtain the promoter region and the non-coding sequence, which may be useful
45 in enhancing transcription and translation of the product.

Employing the subject process, a number of previously undiscovered signal sequences were isolated and demonstrated to be active with genes other than the wild-type gene. These signal sequences encode for amino acid sequences as set forth in the Experimental Section.

50 These signal sequences may be joined to any gene of interest for secretion, particularly in a *Bacillus* host. The sequences may be manipulated in a variety of ways. The 3' proximal sequence may be modified for introduction of a convenient restriction site if necessary. One or more codons may be added to the processing site to provide a convenient restriction site. Usually, the number of codons will be fewer than five, more usually fewer than six, generally at least two, where the codons may act in conjunction with nucleic acids present in the processing site for defining the recognition site. Where a restriction site is
55 upstream from the processing site, an adapter can be prepared which would recreate the necessary codons encoding the processing site. In addition, the adapter may extend into the gene of interest, extending to a convenient restriction site and allowing for the entire construct to be in proper reading frame.

The various fragments may be ligated together in accordance with conventional ways to provide for an expression construct which results in efficient secretion and maturation of the final product. The expression product may be inserted into an appropriate vector, which may contain a transcriptional and translational initiation region, or such region may be associated with the signal sequence, so that the expression construct may include the necessary transcriptional and translational initiation and termination regions. Thus, the construct may be inserted into a vector which allows for replication and selection, as previously described.

A Bacillus host may be used for the production of a wide variety of products, both prokaryotic and eukaryotic. These products may include a variety of enzymes, such as prochymosin, alpha- and beta-amylases, proteases, lipases, esterases, cellulases, mammalian proteins, which include growth factors, plasminogen activators, lymphokines, blood factors, interferons, colony stimulating factors, endorphins, serum albumins, hormones, and receptors.

By virtue of the subject invention, the products may be conveniently processed and secreted into the supernatant, where they may be isolated and purified.

In carrying out the subject invention, particular vectors were prepared. The specific embodiments, i.e. the construction of pGPA11, pGPB11 and pGPP11 is shown in Fig. 1. These vectors were constructed using plasmids pHP12 and pHP14 (see Fig. 2) which are shuttle E. coli -B. subtilis vectors (4.2 kb. Em^R) containing the Em^R gene from pGK13, the origin of replication and the lacZ region from pUC9 and pUC13, respectively, and a 1.6-kb fragment from the cryptic B. subtilis plasmid pTA1060 containing the replication functions.

Plasmid pGPA11 was constructed by inserting a 1.6 kb PstI-HindIII fragment from pGK13-alpha amy into PstI + HindIII digested pHP14. This fragment carries the alpha-amylase gene from B. licheniformis, but lacks the coding region for the first 26 N-terminal amino acid residues of the native signal peptide (29 amino acids in total). Therefore, this vector still contains the original processing site of the alpha-amylase gene.

Plasmid pGPP11 contains a 1.2 kb PstI-HindIII fragment from pRW101 carrying most of the penicillinase gene from B. licheniformis, but lacks the coding region for the first 13 N-terminal amino acid residues (the entire hydrophilic region) of the signal peptide. This fragment was fused to PstI + HindIII digested pHP14.

Plasmid pGPB11 was constructed by inserting into the HindIII site of pHP14 a 1.5 kb HindIII fragment carrying the E. coli beta-lactamase gene from pKTH74 which lacks the entire signal sequence encoding region, including the original processing site.

In a specific embodiment of the invention these three recombinant vectors were used to isolate secretory signal coding regions, by shotgun cloning of restriction fragments of chromosomal B. subtilis DNA into the multicloning sites of the respective vectors. However, as can be seen in Fig. 3, in pGPA11, pGPB11 and pGPP11 the MCS contains an amber (TAG) nonsense codon (at the XbaI site) in reading frame of the structural genes. Since this limits the number of sites in the MCS which can be used for shotgun experiments, in another embodiment this amber codon was eliminated by digesting the plasmids with PstI, followed by removal of the 3' protruding sticky ends by T4 DNA polymerase. The resulting vectors were designated pGPA14, pGPB14, and pGPP14, respectively (see Fig. 3).

In still another embodiment of the invention derivatives of plasmids pGPA14, pGPB14, and pGPP14 were constructed in which the 278 bp EcoRI fragment from pPL608, containing a SPO2 promoter, was cloned into the unique EcoRI site of the MCS. It will be appreciated that any fragment derived from Bacillus phage SPO2 DNA with transcriptional initiation activity may be used. These vectors have the advantage over the original ones in that they allow detection of additional secretory signal coding fragments, namely those which lack promoters.

The invention is further illustrated by the following examples which must be regarded as illustrations only, and in no way as limitations.

EXPERIMENTAL SECTION

Abbreviations used

Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Km^R, kanamycin resistance; MCS, multiple cloning site; bp, base pairs; kb, kilo base pairs.

Media and Plates

TY media contained (per liter): 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl; pH 7.4. Minimal media used in the competence regimen for *B. subtilis* (8G-5) were as described by S. Bron and G. Venema, Mutat. Res. 15 (1972) 1-10. Media used in protoplast transformation of *B. subtilis* were as described by S. Chang and S.N. Cohen, Mol. Gen. Genet. 168 (1979) 111-115. Media were supplemented with selective antibiotics as follows: for *B. subtilis*: Cm, 5 µg/ml; Em, 1 µg/ml and for *E. coli*: Amp, 25 µg/ml; Em, 1 µg/ml; unless stated otherwise.

(Bio)Chemicals

Restriction enzymes, DNA polymerase I, T4 DNA polymerase, Bal 31, mung bean nuclease and T4 DNA ligase, obtained from Boehringer, Mannheim (FRG), or from New England Biolabs (Beverly, Mass.). were used as recommended by the suppliers.

In the Examples the general methods of analysis were conducted as follows.

Gel-Electrophoresis

Agarose gel-electrophoresis was carried out at 10 to 50 mA on 10 cm x 6.5 cm x 0.5 cm horizontal slab gels containing 0.8 to 2.0% agarose (Bio-Rad, Richmond, Calif.). Electrophoresis buffer consisted of 89 mM Tris-HCl, 89 mM boric acid, and 10 mM EDTA, pH 8.3. Ethidium bromide was added to 1 µg/ml to stain the DNA.

Assays for Alpha-Amylase Activity

a) in solution.

The substrate solution in this case contained 60 mg Starch Azure (Sigma) which was suspended in 10 ml 20 mM potassium phosphate, pH 7.5, 50 mM NaCl, and heated for 1 min at 100°C. The mixture was centrifuged for 5 min at 5000 rpm and the pellet was resuspended in 10 ml 20 mM K-phosphate pH 7.5, 50 mM NaCl. Subsequently, 1.5 ml portions of the starch suspension were incubated with appropriate amounts of the enzyme solution and incubated for 10 to 60 min at 37°C in a waterbath shaker (250 rpm). The reactions were stopped by centrifugation for 5 min at 5000 rpm in an Eppendorf centrifuge and then the absorbance at 595 nm of the supernatants was determined. One unit of alpha-amylase was defined as the amount of enzyme that increased the absorbance with 1.0 in 1 h at 37°C.

b) on plates.

E. coli colonies were grown on TY plates supplemented with 1% soluble starch. *B. subtilis* colonies were grown on minimal agar plates without glucose, supplemented with 1% soluble starch. After pouring iodine reagent (0.3% I₂ + 0.6% KI) on the plates, clear halos appeared around the alpha-amylase producing colonies.

c) in SDS-Polyacrylamide gels.

Sodium dodecyl sulfate-polyacrylamide (SDS-PAA) gel electrophoresis was performed on slab gels by the method of Laemmli (U.K. Laemmli, Nature 227 (1970) 680-685). To detect alpha-amylase activity stacking and separating gels contained 0.25% soluble starch. After electrophoresis the gels were washed in 1.0 M Tris/HCl pH 6.8 + 0.25% starch (four times for 30 min) in order to remove the SDS and allow renaturation of the proteins. Subsequently the gels were incubated for 2 h at 65°C (or overnight at 37°C) in the same buffer. Alpha-amylase activity was visualized as clear zones after the starch in the gels was stained blue with 0.5% I₂ + 1.5% KI.

Assays for Beta-Lactamase Activitya) in solution.

Beta-lactamase activity in *B. subtilis* culture supernatants was determined spectrophotometrically using nitrocefin (Becton Dickinson B.V., Amersfoort, Netherlands) according to the method of C.H. O'Callaghan et al., Antimicrob. Ag. Chemother. 1 (1972) 283-288. To prepare the substrate solution 5 mg nitrocefin was dissolved in 0.5 ml dimethylsulfoxide and subsequently diluted to 10 ml with 100 mM K-phosphate pH 7.0. Assay mixtures contained 0.3 ml of the substrate solution, 2.7 ml K-phosphate buffer pH 7.0 and an appropriate amount (25-500 μ l) of the enzyme solution and were incubated for 5 to 10 min at room temperature. One unit of beta-lactamase was defined as the amount of enzyme that increases the absorbance at 486 nm with 0.001 in 1 min at room temperature.

b) on plates.

E. coli colonies producing beta-lactamase were selected directly by plating on TY agar supplemented with Amp (2 to 20 μ g/ml).

B. subtilis colonies, which cannot be selected in this way, were plated on TY or minimal agar supplemented with 0.75% polyvinylalcohol. Beta-lactamase producing colonies were detected as those developing halos after the addition of iodine reagent (0.3% I_2 + 0.6% KI) for 1 min, followed by incubation with 5% penicillin G in 50 mM Na-phosphate, pH 6.4.

25 Originating plasmids

pKTH74 *E. coli* plasmid, 5.8 kb, Amp^R, TC^R, described by I. Palva et al., Proc. Natl. Acad. Sci USA 79 - (1982) 5582-5586.

pGK13 *Streptococcus cemoris* plasmid, derivative of pGK12 4.8 kb, Cm^R, Em^R, described by Kok et al. (1984), lab. collection, University of Groningen, Netherlands.

pGK13-alpha-amylase: pGK13, carrying the alpha-amylase gene of *B. licheniformis*, 7.8 kb, Cm^R, Em^R, alpha-amylase⁺, lab. collection, University of Groningen, Netherlands.

pRW101 bifunctional replicon, carrying the penicillinase gene of *B. licheniformis*, 7.0 kb, Km^R (*B. subt.*), Amp^R(*E. coli*), described by P.S.F. Mezes et al., J. Biol. Chem. 258 (1983) 11211-11218.

pUC9 *E. coli* plasmid, 2.7 kb, Amp^R, described by J. Viera and J. Messing, Gene 19 (1982) 259.

pUC13 *E. coli* plasmid, 2.7 kb, Amp^R, described by J. Messing, Methods Enzymol. 101 (1983) 20-78.

pPL608 *B. subtilis* plasmid, 5.0 kb, Km^R, Cm^R, described by D.M. Williams et al., J. Bacteriol. 146 - (1981), 1162-1165.

pTA1060 cryptic *B. subtilis* plasmid, 7.8 kb, described by T. Uozumi et al., J. Bacteriol. 142 (1980) 315-318.

Example !45 DNA Preparations

Chromosomal DNA was extracted from *B. subtilis* (8G-5) as described by S. Bron and G. Venema, Mutat. Res. 15 (1972) 1-10. Preparation amounts of plasmid DNA were obtained according to the alkaline lysis procedure described by T. Maniatis et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory (1982) p. 90. Selected restriction fragments were obtained by separation on 0.8% or 2.0% agarose gels, followed by extraction and purification on DEAE NA-45 membranes (Schleicher & Schuell, Dassel, FRG) as indicated by the manufacturer. The analytical "miniprep" procedure described by D. Ish-Horowicz and J. F. Burke, Nucl. Acids Res. 9 (1981) 2989-2998 was used to extract plasmids from 2.5 ml cultures of *E. coli*. Minipreps from *B. subtilis* were prepared according to the same procedure with the modifications that before being lysed, the cells from 5 ml cultures were washed once by centrifugation with 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 200 mM NaCl. Miniprep DNAs were incubated with 100 μ g/ml of pancreatic RNase (BDH, Poole, England) for 30 min at 37°C.

Example IIMolecular Cloning Procedures

5 Vector molecules and restricted target DNAs were mixed in approximately a 1:2 weight ratio at a total concentration of 100 µg/ml.

For sticky end ligations the mixtures were heated for 5 min at 68°C. Buffer conditions were adjusted to 20 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 50 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM ATP and 50 µg/ml bovine serum albumin (Boehringer Mannheim, FRG). Then approximately 0.2 U (for sticky end) or 1 U (for
10 blunt end ligations) T₄ ligase was added. The ligation mixtures were incubated overnight at 4°C (sticky ends) or overnight at room temperature (blunt ends). Ligated samples were used to transform B. subtilis or E. coli competent cells.

Filling in 3' recessed sticky ends was carried out by incubating the DNA (100 µg/ml) in 50 mM Tris-HCl, pH 7.2, 10 mM MgSO₄, 0.1 mM DTT, 50 µg/ml BSA with 1 U of the Klenow fragment of DNA polymerase I
15 and 0.1 mM of the four dNTPs for 15 min at room temperature, or by incubating the DNA (100 µg/ml) with 1 U of T₄ DNA polymerase for 30 min at 37°C in a buffer containing 33 mM Tris-acetate pH 7.9, 66 mM K-acetate, 10 mM Mg-acetate, 0.5 mM DTT, 0.1 mg/ml BSA and 0.1 mM of the four dNTPs. 3' protruding
termining were digested with T₄ DNA polymerase under the same conditions. For partial digestions with Bal 31 the DNA (100 µg/ml) was incubated for 10 min at 30°C with 1 U of Bal 31 in 20 mM Tris-HCl pH 9.1, 60
20 mM NaCl, 12 mM CaCl₂, 12 mM MgCl₂ and 1 mM EDTA. Bal 31-digested DNAs were treated with T₄ DNA polymerase as described above and subsequently used for ligation.

In order to remove 5'-protruding sticky ends, DNA (100 µg/ml) was incubated for 25 min at 25°C with 0.5 U mung bean nuclease in SI buffer containing: 5 mM Na-acetate pH 4.6, 280 mM NaCl, 4.5 mM ZnSO₄, and 0.5% glycerol.

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Example IIITransformations

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Portions of 0.1 ml of competent B. subtilis cells were exposed to 0.5 to 1 µg of plasmid DNA for 30 min at 37°C in a waterbath shaker. To allow time for expression of antibiotic resistance markers, 0.1 ml of TY medium was added and the suspension was incubated for 1 h at 37°C. Transformants were selected on TY plates supplemented with selective antibiotics (Em, 1 µg/ml; Km, 5 µg/ml). Protoplasts of B. subtilis were
35 transformed and regenerated as described by S. Chang and S.N. Cohen, Mol. Gen. Genet. 168 (1979) 111-115. Transformed protoplasts were plated on DM3 medium containing Em (5 µg/ml). E. coli cells were made competent and were transformed with approximately 1 µg/ml of plasmid DNA by the CaCl₂/cold-shock procedure described by T. Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory (1982) p. 250. After allowing time for expression of antibiotic resistance markers (1 h,
40 37°C), transformants were selected on antibiotic-containing TY plates (Em, 200 µg/ml or Em, 50 µg/ml + Amp, 2 µg/ml).

Example IV

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Shotgun Cloning Procedures for Secretory Signals.

B. subtilis chromosomal DNA was digested with Sau3A, Alu I, HaeIII or RsaI and subsequently ligated with BamHI or SmaI-digested vector molecules (pGPB14 or pGPA14) in a 4:1 weight ratio. Ligated samples
50 were used to transform competent E. coli cells. Transformants were selected by plating on TY plates containing Em (200 µg/ml) + 1% soluble starch (for pGPA14). Plasmid DNAs extracted from the transformants were subsequently used to transform competent B. subtilis cells with selection for Em (1 µg/ml). Transformants were analyzed for their capacity to secrete either alpha-amylase or beta-lactamase into the culture medium as described hereinbefore in the Experimental Section.

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Example VMolecular Cloning of Known Secretory Signal Sequences

5 This Example shows the effects of fusion of the truncated genes to a number of known signal sequences in the correct reading frame, see Figures 4 and 5.

Purified BamHI-PstI fragments from pRW101 and pGK13-alpha-amyl were ligated with BamHI + PstI digested pGPB11. The 300 bp BamHI-PstI fragment from pRW101 contains the promoter and the coding region for the first 13 N-terminal amino acids of the penP signal peptide. The 940 bp BamHI-PstI fragment
 10 from pGK13-alpha-amyl encodes the entire promoter and signal sequence, except for the last 3 amino acids, of alpha-amylase. Since Amp^R transformants cannot be selected directly in B. subtilis (Amp^R was defined as the ability of a single cell to form a colony on ampicillin-containing plates), ligation mixtures were used to transform E. coli BHB2600 cells. The construct pGPB15, in which the truncated beta-lactamase gene is preceded by the promoter and signal sequence coding region of the alpha-amylase gene, yielded a
 15 considerable number of Amp^R E. coli transformants. However, the fusion of the promoter and the hydrophilic part of the pen P signal sequence coding region to the truncated beta-lactamase gene (pGPB16) did not yield Amp^R transformants in E. coli.

These results demonstrate that the appearance of Amp^R transformants in E. coli was dependent on the fusion of a functional signal sequence to the truncated beta-lactamase gene and that the export of beta-lactamase was restored. Moreover, since the wild-type precursor of beta-lactamase is known to be inactive
 20 (P.C. Tai et al., Anal. Biochem. 144 (1985) 199-203 and R. Roggenkamp et al., J. Biol. Chem. 260 (1985) 1508-1512) the appearance of Amp^R transformants (= beta-lactamase activity) indicates that the hybrid protein is processed. Furthermore, these results support the assumption that signal sequences may be functionally interchangeable (see also Example IX).

25 Also the experiments with pGPP3 (containing the original penP signal sequence) and pGPP5 (containing the original hydrophobic core of this signal extended with 15 extra amino acids, see for details Fig. 5) revealed that the hybrid signal sequence is functional in E. coli, cf. Table 1.

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TABLE 1
Properties of Various Gene Fusions in
B. subtilis and E. coli

<u>B. subtilis</u>	<u>E. coli</u>
plasmids halos on plates	plasmids levels of resistance to Amp
pGPB14(SPO2) -	pGPB14(SPO2) 0
pGPB14 -	pGPB14 0
pGPB15 -	pGPB15 A ₂₀ :100%, A ₅₀ :1% A ₁₀₀ :0.1%
pGPP11(SPO2) +	pGPP11(SPO2) "inoculum effect"
pGPP11 -	pGPP11 0
pGPP3 +++	pGPP3 A ₃₀₀ :100%
pGPP5 ++	pGPP5 A ₂₀ :100%, A ₁₀₀ :50% A ₂₀₀ :30%, A ₃₀₀ :20%

B. subtilis cells containing various recombinant plasmids were analyzed for their ability to form halos on plates containing polyvinylalcohol as described on page 17. Overnight cultures of E. coli cells containing the various recombinant plasmids were plated on TY plates supplemented with increasing concentrations of ampicillin. The maximal ampicillin concentration tested was 300 µg/ml. The number of viable cells was expressed as percentage of those on non-selective plates. Plasmid pGPP11 shows a pronounced "inoculum effect" on the level of resistance that can be obtained. This means that a single cell is virtually defenseless against low concentrations of ampicillin, whereas the resistance level increases enormously when larger inocula are used.

From the results shown in Table 1 it appears that in contrast to the control (pGPB11), plasmid pGPB15 rendered E. coli cells resistant to rather high concentrations of Amp. However, in B. subtilis, neither 8G-5 (pGPB15), nor 8G-5 (pGPB14) showed the ability to form halos on polyvinylalcohol plates. The apparent lack of secretion of beta-lactamase by B. subtilis is probably due to the breakdown of the secreted beta-lactamase by extracellular proteases (K. Nakamura et al., Biochem. Biophys. Res. Comm. 128 (1985) 601-

606 and I. Ulmanen et al., J. Bacteriol. 162 (1985) 172-182). It is noteworthy that in contrast to E. coli cells that contain plasmid pGPP3, in which the original penP signal sequence was restored, E. coli cells containing pGPP5, in which the hybrid amylase-penP signal sequence preceded penP, are less resistant to Amp. This phenomenon which is also reflected by a decreased penicillinase production in B. subtilis, most likely can be explained by differences in secretory efficiencies of the two signal sequences.

Example VI

10 Molecular Cloning of Unknown Secretory Signal Sequences

In order to minimize the risk of missing secretory signals by cloning the inserts in incorrect translational reading frames relative to the target genes a number of different restriction enzymes (Sau3A, AluI, HaeIII, Rsa I) were used to cleave the B. subtilis 8G-5 chromosomal DNA. After shotgun cloning of B. subtilis DNA fragments into the various vectors, clones were selected in E. coli, either by their ability to form halos on starch plates (pSPA plasmids) or by their resistance to Amp (pGPB plasmids). Out of the thousands of colonies tested, alpha-amylase positive clones were obtained with efficiencies varying from 0.1 to 0.5%, whereas Amp^R clones were obtained about 5 times less efficiently (0.02-0.1%).

A random selection of 20 potential secretory-proficient clones is presented in Table 2. Restriction analysis showed that inserts of various length had been cloned. Moreover, the various inserts rendered the E. coli cells resistant to different concentrations of Amp. These results clearly demonstrate that this invention permits the isolation of many different secretory signal sequences.

TABLE 2
Properties of Potential Secretary-Proficient Clones
Obtained in E. coli

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plasmid ¹	size of insert (bp) ²	Amp ^R
pSPB4'	710	10
pSPB5'	650	20
pSPB6'	790	10
pSPB8'	670	275
pSPB9'	360	150
pSPB11'	270	5
pSPB12'	830	10
pSPB13'	650	10
pSPB16'	790	50
pSPB19'	420	50
pSPB20'	1500	600
pSPB21	740	50
pSPB23	490	175
pSPB28	280	190
pSPB29	300	5
pSPB30	260	5
pSPB31	420	5
pSPB32	1080	300
pSPB34	370	10
pSPB38	670	20

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- 1) The pSPB clones shown do not contain the SPO2 promoter.
- 2) All pSPB clones contain Sau3A inserts. Since some of the pSPB clones contained partially digested Sau3A fragments, those inserts were redigested with Sau3A and religated in pGPB14 digested with Bam HI. The new clones are indicated with a prime. The size of the inserts was determined by restriction analysis.

The level of resistance to Amp was defined as the maximal concentration of ampicillin to which 100% of the viable cells were resistant. This was determined as described in the legends to Table 1.

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Example VIIAlpha-Amylase Activities Produced by the Recombinant Plasmids in *B. subtilis*.

5 In order to prove that the selected inserts contain coding regions for export functions, a number of randomly chosen recombinant plasmids were transformed to competent *B. subtilis* cells and the transformants obtained were analyzed for alpha-amylase activities in the culture supernatants. The assays of the alpha-amylase activities (Table 3, page 26) show that considerable amounts of alpha-amylase activity could be detected in the culture supernatants of *B. subtilis* cells containing the recombinant plasmids. These results confirm the cloning of secretory signal sequence coding regions.

10 *B. subtilis* 8G-5(pSPA2, pSPA8 and pSPA12), respectively, exhibited alpha-amylase activities which were substantially higher than those produced by *B. subtilis* (pGK13-alpha-amylase) i.e. the plasmid containing the native alpha-amylase secretory signal sequence. Since all alpha-amylase positive transformants were selected with a vector containing the SPO2 promoter, the inserts do not necessarily contain promoter activity. Deletion of the SPO2 promoter in pSPA8, pSPA9, pSPA12, and pSPA42 revealed (Table 3) that some inserts contain promoter activity indeed (pSPA8, pSPA9, pSPA12) and others do not (pSPA42).

15 This observation clearly shows that secretory functions can be selected with a higher efficiency by using selection vectors which contain a promoter sequence upstream of the site used for insertion of target DNA.

20 Said plasmids pSPA2, pSPA8 and pSPA12 were deposited in *Bacillus subtilis* strain 8G-5 on May 2, 1986 under the provisions of the Budapest Treaty with the Centraalbureau voor Schimmelcultures, Oosterstraat 1, NL-3742 SK Baarn, and were allotted the numbers CBS 248.86, CBS 249.86 and CBS 250.86, respectively.

TABLE 3

Amounts of Alpha-Amylase in the Culture Supernatants
of Various *B. subtilis* Clones.

plasmid ¹	size of insert (bp) ²	alpha-amylase activity in the culture supernatant (U/ml) of <i>B. subtilis</i>
pSPA2 *)	730	525
pSPA3 *)	340	345
pSPA8(-SPO2)	340	175
pSPA9	1400	45
pSPA9(-SPO2)	1400	2
pSPA12 *)	700	270
pSPA12(-SPO2)	700	15
pSPA13	1200	150
pSPA26	210	3
pSPA31	50	60
pSPA42	400	4
pSPA42(-SPO2)	400	0
pGPA14		0
pGK13-alpha-amylase		100

- 1) All pSPA clones shown contain the SPO2 promoter, except where otherwise stated.
2) pSPA2 contains an AluI insert, pSPA8 contains a HaeIII insert, and pSPA9, 12, 13, 26, 31 and 42 contain a RsaI insert.

* The plasmids pSPA2, pSPA3 and pSPA12 were deposited with the CBS under Nos. 248.86, 249.86 and 250.86, respectively

The *B. subtilis* cells were grown for one day in TY medium and the alpha-amylase activities in the culture supernatants were determined as described hereinbefore on page 16.

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Example VIIIBeta-Lactamase Activities Produced by the Recombinant Plasmids in *B. subtilis*

5 A comparative study was made on the beta-lactamase activities in the culture supernatants of *B. subtilis* cells (strain 8G-5) carrying various recombinant plasmids, either on polyvinylalcohol plates or by the nitrocefin assay, after growing the cells overnight in rich media. However, significant beta-lactamase activity could not be detected by using these methods. This result is in accordance with the results described in Example V for 8G-5(pGPB15). Most likely the absence of activity is caused by the sensitivity of the beta-lactamase to the *B. subtilis* exo-proteases (K. Nakamura et al., Biochem. Biophys. Res. Comm. 128 (1985) 601-606, I. Ulmanen et al., J. Bacteriol. 162 (1985) 172-182, I. Palva et al., Proc. Natl. Acad. Sci. USA 79 (1982) 5582-5586). This could be demonstrated by an investigation of the betalactamase production in minimal versus rich media (see Fig. 6). In TY medium, the amount of beta-lactamase increased during the logarithmic and early stationary phases of growth.

10 However, the amount of beta-lactamase was decreasing after prolonged growth and was completely lost in overnight cultures. In contrast, the amount of beta-lactamase remained constant up to 24 h in minimal medium. These results support the idea that, at least in TY medium, beta-lactamase is unstable, probably due to proteolysis.

15 The amount of beta-lactamase in the supernatant of some pSPB clones is given in Table 4.

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TABLE 4
Amounts of Beta-Lactamase in the Culture
Supernatants of Various *B. subtilis* Clones

Plasmid	<i>B. subtilis</i> beta-lactamase activity (U/ml)
pSPB4'	630
pSPB8'	800
pSPB9'	16
pSPB19'	230
pSPB20'	585
pSPB23	9
pSPB28	17
pSPB32	78

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The amount of beta-lactamase was determined in minimal medium as described hereinbefore on page 17.

Example IXThe interchangeability of the Cloned Secretory Signals

5 This example shows that the secretory signal sequences detected with pGPA14 are functional in pGPB14 and vice versa (see Table 5). Most constructed pSPB-A vectors, containing the beta-lactamase gene preceded by inserts derived from pSPA clones, conferred resistance to ampicillin in E. coli. Moreover, the same plasmids produced considerable levels of beta-lactamase activity in B. subtilis. Furthermore, the pSPA-B clones, in which the alpha-amylase gene is preceded by inserts originally selected in pGPB14,
10 produced and secreted alpha-amylase (see Table 5).

However, a comparison of Tables 5 and 3 shows that in B. subtilis the relative order of the levels of alpha-amylase activities with the original pSPA vectors (Table 3) and those of the beta-lactamase with the interchanged pSPB-A vectors (Table 5) were not identical. Qualitatively similar results were obtained in the reciprocal experiment (Tables 5 and 4). Since in this comparison no differences at the transcriptional and
15 translational level existed, and the same secretory signals were used, these results suggest that the efficiency of protein secretion in B. subtilis was affected both by the nature of the secretory signal sequences as well as by the mature parts of the proteins.

Furthermore, the levels of beta-lactamase activities in the supernatants of B. subtilis cells containing pSPB-A vectors (see Table 5) were on average higher (sometimes even 10-30 times) than those in the
20 supernatants of B. subtilis cells containing pSPB vectors (see Table 4). Moreover the pSPA vectors generally gave slightly higher levels of alpha-amylase than the pSPA-B vectors.

In conclusion, these results show that all the cloned secretory signal sequences tested, were functionally interchangeable between the various vectors and the results suggest that with the two probe vectors different "classes" of secretory signals have been picked up.

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TABLE 5
Interchangeability of Secretory Signals

Plasmid	<u>E. coli</u> amp ^R	<u>Bacillus subtilis</u> beta-lactamase activity U/ml		Plasmid	<u>Bacillus subtilis</u> alpha-amylase activity U/ml	
pSPB-A2 (-SPO2)	175	27.000		pSPA-B4'		123
pSPB-A2 (+SPO2)	300	20.000		pSPA-B8'		16
pSPB-A8 (-SPO2)	300	495		pSPA-B9'		5
pSPB-A9 (+SPO2)	300	2.900		pSPA-B19'		0.6
pSPB-A12 (-SPO2)	10	34		pSPA-B20'		20
pSPB-A13 (-SPO2)	300	4.200		pSPA-B23		3.8
pSPB-A13 (+SPO2)	300	1.600		pSPA-B28		37
pSPB-A26 (+SPO2)	0	880		pSPA-B32		96
pSPB-A31 (+SPO2)	100	720		pSPA-B9' (+SPO2)		82
pSPB-A42 (+SPO2)	20	710		pSPA-B19' (+SPO2)		12
				pSPA-B20' (+SPO2)		40
				pSPA-B23 (+SPO2)		57

The amp^R level of E. coli cells was determined as described in the legends of Tables 1 and 2. Beta-lactamase activities of B. subtilis cells were determined with nitrocefin as described hereinbefore on page 17. Alpha-amylase activities of B. subtilis cells were determined as described hereinbefore on page 16.

Example X

Secretion Versus Lysis

The results presented in the previous examples clearly demonstrate that the vectors pGPB14 and pGPA14 can be used to select for secretory coding regions, which function both in E. coli and in B. subtilis. To exclude the possibility that the enzymatic activities measured in B. subtilis were the result of cell lysis rather than of secretion, the cellular location of alpha-amylase produced by pSPA2 and pSPA8 was compared with the location of a cytoplasmic marker protein, for which isocitrate dehydrogenase (ICDH) was

chosen. At first it was tested whether ICDH itself was stable in various media (see Table 6). After 24 h of incubation at 37°C in minimal medium or in the supernatant of a *B. subtilis* overnight culture in this medium, 80% of the original ICDH activity was still present. However, by incubation in TY medium the activity was completely lost after 3 to 5 hr at 37°C.

TABLE 6

Stability of Isocitrate Dehydrogenase (ICDH)
Activity in Various Media

time (h)	TY medium	supernatant (TY medium)	minimal medium	supernatant (minimal medium)
0	100	100	100	100
1	75	77	123	100
2	40	34	116	126
3	14	15	116	125
5	0	0	118	126
24	0	0	76	84

10 ml portions of an overnight culture of *B. subtilis* strain 8G-5 (or DB104) in either TY, or minimal medium were centrifuged for 10 min at 6000 rpm. The cells were washed twice by centrifugation. The pellets were resuspended in 5 ml starvation medium, containing 200 µg/ml lysozyme, and incubated for 15 min at 37°C. 2.5 ml portions of the lysed cells were subsequently mixed with 2.5 ml portions of either TY medium, supernatant of a TY culture, minimal medium or culture supernatant of a culture grown in minimal, and incubated at 37°C. As a function of time the ICDH activities are given as percentages of the original ICDH activity.

Similar results were obtained with supernatants of *B. subtilis* cultures of 8G-5 and DB104 (protease deficient strain, F. Kawamura and R.H. Doi, J. Bacteriol. 160 (1984) 442-444). These results indicate that ICDH can be used as a cytoplasmic marker in minimal, but not in TY medium.

Table 7 shows that the alpha-amylase activity was predominantly found in the culture supernatants whereas most if not all of the ICDH activity was associated with the cell pellets. These results strongly support the conclusion that the alpha-amylase activities measured in the supernatants were the result of secretion rather than of cell lysis.

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TABLE 7

Alpha-Amylase and ICDH Activities in Cell Pellets and
Culture Supernatants of two *B. subtilis* Clones.

plasmid	alpha-amylase activity (U/ml)		ICDH activity (U/ml)	
	supernatant	pellet	supernatant	pellet
pSPA2	100	1	3.9	287
pSPA8	83	8	23	122

Cultures were grown overnight in minimal medium without glucose. 1 ml portions were centrifuged for 3 min in an Eppendorf centrifuge. The cells were washed twice with 100 mM K-phosphate, pH 8.0. The pellets were suspended in 0.5 ml 100 mM K-phosphate, pH 8.0, 200 µg/ml lysozyme and incubated for 15 min at 37°C. The culture supernatants were filtered through a 0.45 µm filter in order to remove cells and debris. Subsequently, the alpha-amylase and ICDH activities were determined.

Example XI

Alpha-Amylase Activities in SDS-Polyacrylamide Gels.

The distribution of alpha-amylase activity in culture supernatants in cell pellets of a number of pSPA clones on SDS-polyacrylamide (PAA) gels was examined. Purified alpha-amylase from *B. licheniformis* was used as a control to determine the molecular weight position of the mature alpha-amylase. The molecular weight positions of the alpha-amylases isolated from the culture supernatants of 8G-5(pGK13-amy) and 8G-5(pSPA2, pSPA8, pSPA9) seemed to be identical to that of the control indicating that in all constructs cleavage of the secretory signal function had occurred at about the same processing site of alpha-amylase. Extracts of cell pellets from 8G-5(pSPA2 and pSPA8) showed alpha-amylase bands at higher molecular weight positions than found in the supernatants. The precursor in 8G-5(pSPA2) is of higher molecular weight than that expressed in 8G-5(pSPA8). It would appear that some of the precursor forms of the *B. licheniformis* alpha-amylase are enzymatically active. Since the precursor bands could be detected immunologically as well as enzymatically with the same intensities relative to the mature products, it would appear that the specific activity of the precursor forms is similar to that of the mature product. Since in 8G-5(pSPA2, pSPA8) under steady state conditions precursor forms could be detected, the rate of secretion and/or processing of the alpha-amylase in these fusions appear to be delayed as compared to the wild-type. No precursor forms could be detected in the cell pellets of 8G-5(pSPA9, pSPA12, pSPA13) in the gels.

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Example XIIThe Cell Associated Alpha-Amylase

5 The amounts of alpha-amylase activities associated with the cell pellets were determined. The results are shown in Table 8.

TABLE 8

Cell-Associated Enzymatic Activities

plasmid	% cell-associated alpha-amylase activity
pSPA2 (+SPO2)	0.6
pSPA8 (+SPO2)	14
pSPA9 (+SPO2)	6
pSPA12 (-SPO2)	1.3
pSPA13 (+SPO2)	1.4
pSPA26 (+SPO2)	73
pSPA31 (+SPO2)	2.5
pSPA42 (+SPO2)	12
pSPA-B4' (-SPO2)	4
pSPA-B8' (-SPO2)	13
pSPA-B9' (-SPO2)	1
pSPA-B9' (+SPO2)	2
pSPA-B19' (-SPO2)	0
pSPA-B19' (+SPO2)	2
pSPA-B20' (-SPO2)	21
pSPA-B20' (+SPO2)	17
pSPA-B23 (-SPO2)	33
pSPA-B23 (+SPO2)	19
pSPA-B28 (-SPO2)	2
pSPA-B32 (-SPO2)	1
pGK13-amy	0

55 B. subtilis cells were grown overnight in TY medium. Alpha-amylase activities in supernatants and cell pellets were determined as described hereinbefore on page 16.

In cells with pGK13-amy, containing the wild-type alpha-amylase gene, no activity could be detected in extracts of the cell pellets. With the exception of 8G-5(pSPA-B19'), reproducible amounts of alpha-amylase activity were associated with the cell pellets. As evidenced by the above results, a number of plasmids provide relatively large amounts of cell associated alpha-amylase. In the presence of the SPO2 promoter, the amounts of the alpha-amylase activities in the supernatants of *B. subtilis* cultures containing the various plasmids was increased. Despite this increase, the relative amount of cell-associated alpha-amylase was not altered.

10 Example XIII

Secretory Signal Sequences of Various Recombinant Plasmids

Inserts of pSPB4' to pSPB20', pSPB21 to SPB38 and pSPA2 to pSPA42 were sequenced according to the dideoxy method of Sanger and analyzed for the presence of secretory signal sequences. By matching the three reading frames with the known reading frame of alpha-amylase or beta-lactamase, the proper reading frame was determined. Consequently, the length of the precursor (the secretory signal sequence plus mature enzyme) was compared with data obtained with an *in vitro* transcription-translation system in order to support the validity of the sequence. The following table indicates a number of the sequences.

TABLE 9

<u>Insert Of</u>	<u>Sequence</u>
pSPA2	Met arg+ lys+ ser leu ile thr leu gly leu ala ser val ile gly thr ser ser phe leu ile pro phe thr ser lys+ thr ala ser ala glu- thr leu asp- glu- lys+ lys+ gln lys+ ile glu- ser lys+ gln ser glu- val ala ser ser ile glu- ala lys+ glu- lys+ glu- leu thr glu- \int^* gly asp- pro leu glu- ser thr ala ala ala
pSPA8	Met lys+ lys+ met leu val val leu leu phe ser ala leu leu leu asn gly cys gly ser gly glu- ser lys+ ala asn thr ala glu- thr pro glu- val leu asp- val lys+ leu thr gly \int gly asp- pro leu glu- ser thr ala ala ala

pSPA12 Met ile arg+ gly ile leu ile ala val leu gly ile
 ala ile val gly \int gly asp- pro leu glu- ser thr
 ala ala ala
 5

pSPA13 Met leu lys+ lys+ val ile leu ala ala phe ile
 leu val gly ser \int gly asp- pro leu glu- ser thr
 ala ala ala
 10

pSPA26 Met ser gln gln his+ asp- tyr val ile gly lys+
 asn ala val ile glu- thr leu lys+ ser asp- arg+
 leu asp- leu phe pro leu leu arg+ leu thr lys+
 lys+ pro lys+ val gln thr gly ile asp- thr leu
 leu pro asp- tyr lys+ lys+ gln \int gly asp- pro leu
 glu- ser thr ala ala ala
 15
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pSPA31 Met asp- glu- val his+ glu- glu- glu- |** glu-
 phe glu- leu ala pro gly leu phe ile leu leu phe
 leu phe val met ala val ile gly \int gly asp- pro
 leu glu- ser thr ala ala ala
 25
 30

pSPA42 Met leu lys+ arg+ thr ser phe val ser ser leu
 phe ile ser ser ala val leu leu ser ile leu leu
 pro ser gly leu ser his+ thr leu ser ala lys+
 gly thr asn lys+ asn asn leu phe phe phe asp-
 thr glu- thr thr gly leu gly gly gly ala gly asn
 thr ile phe leu leu gly his+ ala arg+ val tyr
 glu- asp- arg+ val thr val lys+ gln his+ leu leu
 pro lys+ pro gly asn glu- val ala leu tyr
 gln ser phe leu ser glu- val asp- ile thr ser
 leu val thr tyr asn gly lys+ ala phe asp- trp \int
 gly asp-pro leu glu- ser thr ala ala ala
 35
 40
 45

pSPB4' Met lys+ ile ser arg+ ile leu leu ala ala val
 ile leu ser ser val phe ser ile thr tyr leu gln
 ser asp- leu gly trp phe ala lys+ glu- gly gln
 met asp- glu- thr phe thr lys+ ala ala phe lys+
 leu lys+ thr gly glu- val ser asp- \int pro leu glu-
 ser thr ala gln ala cys
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pSPB9' Met lys+ gln thr val leu leu leu phe thr ala leu
 phe leu ser gly cys ser val ala ser ala asp-
 5 asp- ser val pro arg+ phe thr glu- glu- gly lys+
 tyr ile gly ser ala asp- ∫ pro leu glu- ser thr
 ala gln ala cys

10 pSPB19' Met lys+ lys+ leu val phe gly leu leu ala ile
 val leu phe gly cys gly leu tyr ile tyr hist+ val
 trp phe gly asp- ∫ pro leu glu- ser thr ala gln
 15 ala cys

pSPB20' Met leu lys+ lys+ cys ile leu leu val phe leu
 20 cys val gly leu ile gly leu ile gly cys ser
 lys+ thr asp- ser pro glu- asp- ∫ pro leu glu-
 ser thr ala gln ala cys

25 pSPB23 Met arg+ lys+ trp ile ala ala ala gly leu ala
 tyr val leu tyr gly leu phe phe tyr trp tyr phe
 phe leu ser gly asp- ser ala ile pro glu- ala
 30 val lys+ gly thr gln ala asp- ∫ pro leu glu- ser
 thr ala gln ala cys

35 pSPB28 Met pro ile lys+ lys+ lys+ val met met cys leu
 ala val thr leu val phe gly ser met ser phe pro
 thr leu thr asn ser gly gly phe lys+ glu- ser
 40 thr asp- ∫ pro leu glu- ser thr ala gln ala cys

pSPB32 Met lys+ leu val pro arg+ phe arg+ lys+ gln trp
 45 phe ala tyr leu thr val leu cys leu ala leu ala
 ala ala val ser phe gly val pro ala lys+ ala ala
 glu- asn pro gln thr ser val ser asn thr gly
 lys+ glu- ala asp- ala thr lys+ asn gln thr ser
 50 lys+ ala asp- ∫ pro leu glu- ser thr ala gln ala
 cys

55 * The amino acids behind this line ∫ are from the multiple
 cloning site of the plasmid.

** The amino acids before this line | are from the SPO2
 fragment.

By using hydrophobicity plotting of Hopp and Woods, Proc. Natl. Acad. Sci. USA 78 (1981) 3824-3828, it was demonstrated that most secretory signals have a hydrophilic head (positively charged) and a hydrophobic core. Several of the secretory signal sequences, .g. pSPA2 and pSPB32, differ from signal sequences which are known from the literature as being significantly longer. A number of those sequences improve secretion as demonstrated with pSPA2.

As is evidenced by the above results, the subject method provides for a convenient and efficient system for isolating signal sequences from hosts and evaluating their efficiency in a rapid and convenient manner. The signal sequences may then be employed with a wide variety of genes other than the wild-type, to provide for efficient production of an open reading frame of interest. In this manner, a variety of hosts, particularly unicellular microorganism hosts, more particularly prokaryotic hosts, may be screened to identify the sequences which may find use for the production of various peptides in unicellular microorganisms.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Claims

1. A plasmid useful for detecting secretory signal sequences from DNA fragments of sequences of an unicellular microorganism cell host of interest, said plasmid comprising:
a replication system functional in an expression cell host;
a multiple cloning site having at least two restriction sites unique to said plasmid;
an open reading frame encoding an enzyme, capable of being detected upon secretion, contiguous to said multiple cloning site and in reading frame with said two unique restrictions sites.
2. A plasmid according to Claim 1, wherein said expression cell host is a Bacillus.
3. A plasmid according to Claim 1 or 2, further comprising a replication system functional in a cloning cell host.
4. A plasmid according to Claim 3, wherein said cloning cell host is E. coli.
5. A plasmid according to any one of Claims 1-4, wherein said unicellular microorganism cell host is a Bacillus.
6. A plasmid according to any one of Claims 1-5, wherein said enzyme is alpha-amylase, beta-lactamase or penicillinase.
7. A plasmid according to any one of Claims 1-6, comprising a transcriptional initiation region upstream from said multiple cloning site, where the direction of transcription is from said initiation region through said multiple cloning site to said enzyme, and which initiation region is functional at least in said expression cell host.
8. A plasmid according to Claim 7, wherein said transcriptional initiation region is a Bacillus SPO, transcriptional initiation region.
9. A plasmid according to any one of Claims 1-6, comprising a DNA fragment from said cell host of interest inserted or substituted into said multiple cloning site at at least one unique restriction site, and which initiation region is functional at least in said expression cell host.
10. A plasmid according to Claim 7 or 8, comprising a DNA fragment from said cell host of interest inserted or substituted into said multiple cloning site at at least one unique restriction site.
11. A plasmid selected from the group consisting of pGPA14, pGPB14, pGPP14 and deposited with the CBS under nos. 245.86, 246.86, 247.86, respectively.
12. A plasmid as identified in any of Tables 2 to 5 of the specification by the designation pSPA or pSPB.
13. A DNA sequence flanked by and in proper reading frame with a non-wild type open reading frame encoding a secretory signal sequence having the amino acid sequence:

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Insert OfSequence

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pSPA2

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Met arg+ lys+ ser leu ile thr leu gly leu ala
 ser val ile gly thr ser ser phe leu ile pro phe
 thr ser lys+ thr ala ser ala glu- thr leu asp-
 glu- lys+ lys+ gln lys+ ile glu- ser lys+ gln
 ser glu- val ala ser ser ile glu- ala lys+ glu-
 lys+ glu- leu thr glu- \int^* gly asp- pro leu glu-
 ser thr ala ala ala

pSPA8 Met lys+ lys+ met leu val val leu leu phe ser
 ala leu leu leu asn gly cys gly ser gly glu- ser
 5 lys+ ala asn thr ala glu- thr pro glu- val leu
 asp- val lys+ leu thr gly \int gly asp- pro leu glu-
 ser thr ala ala ala

10 pSPA12 Met ile arg+ gly ile leu ile ala val leu gly ile
 ala ile val gly \int gly asp- pro leu glu- ser thr
 ala ala ala

15 pSPA13 Met leu lys+ lys+ val ile leu ala ala phe ile
 leu val gly ser \int gly asp- pro leu glu- ser thr
 20 ala ala ala

pSPA26 Met ser gln gln his+ asp- tyr val ile gly lys+
 25 asn ala val ile glu- thr leu lys+ ser asp- arg+
 leu asp- leu phe pro leu leu arg+ leu thr lys+
 lys+ pro lys+ val gln thr gly ile asp- thr leu
 30 leu pro asp- tyr lys+ lys+ gln \int gly asp- pro leu
 glu- ser thr ala ala ala

pSPA31 Met asp- glu- val his+ glu- glu- glu- |** glu-
 35 phe glu- leu ala pro gly leu phe ile leu leu phe
 leu phe val met ala val ile gly \int gly asp- pro
 leu glu- ser thr ala ala ala

40 pSPA42 Met leu lys+ arg+ thr ser phe val ser ser leu
 phe ile ser ser ala val leu leu ser ile leu leu
 45 pro ser gly leu ser his+ thr leu ser ala lys+
 gly thr asn lys+ asn asn leu phe phe phe asp-
 thr glu- thr thr gly leu gly gly gly ala gly
 50 asn thr ile phe leu leu gly his+ ala arg+ val
 tyr glu- asp- arg+ val thr val lys+ gln his+ leu
 leu pro lys+ pro gly asn glu- val ala leu tyr
 gln ser phe leu ser glu- val asp- ile thr ser
 55 leu val thr tyr asn gly lys+ ala phe asp- trp
 \int gly asp- pro leu glu- ser thr ala ala ala

5 pSPB4' Met lys+ ile ser arg+ ile leu leu ala ala val
 ile leu ser ser val phe ser ile thr tyr leu gln
 ser asp- leu gly trp phe ala lys+ glu- gly gln
 10 met asp- glu- thr phe thr lys+ ala ala phe lys+
 leu lys+ thr gly glu- val ser asp- \int pro leu glu-
 ser thr ala gln ala cys

15 pSPB9' Met lys+ gln thr val leu leu leu phe thr ala leu
 phe leu ser gly cys ser val ala ser ala asp-
 asp- ser val pro arg+ phe thr glu- glu- gly lys+
 20 tyr ile gly ser ala asp- \int pro leu glu- ser thr
 ala gln ala cys

25 pSPB19' Met lys+ lys+ leu val phe gly leu leu ala ile
 val leu phe gly cys gly leu tyr ile tyr hist+ val
 trp phe gly asp- \int pro leu glu- ser thr ala gln
 30 ala cys

pSPB20' Met leu lys+ lys+ cys ile leu leu val phe leu
 cys val gly leu ile gly leu ile gly cys ser
 35 lys+ thr asp- ser pro glu- asp- \int pro leu glu-
 ser thr ala gln ala cys

40 pSPB23 Met arg+ lys+ trp ile ala ala ala gly leu ala
 tyr val leu tyr gly leu phe phe tyr trp tyr phe
 phe leu ser gly asp- ser ala ile pro glu- ala
 45 val lys+ gly thr gln ala asp- \int pro leu glu- ser
 thr ala gln ala cys

pSPB28 Met pro ile lys+ lys+ lys+ val met met cys leu
 50 ala val thr leu val phe gly ser met ser phe pro
 thr leu thr asn ser gly gly phe lys+ glu- ser
 thr asp- \int pro leu glu- ser thr ala gln ala cys
 55

pSPB32

Met lys+ leu val pro arg+ phe arg+ lys+ gln trp
 phe ala tyr leu thr val leu cys leu ala leu ala
 5 ala ala val ser phe gly val pro ala lys+ ala ala
 glu- asn pro gln thr ser val ser asn thr gly
 lys+ glu- ala asp- ala thr lys+ asn gln thr ser
 10 lys+ ala asp- pro leu glu- ser thr ala gln ala
 cys

* The amino acids behind this line are from the
 multiple cloning site of the plasmid.

15 ** The amino acids before this line are from the SPO₂
 fragment.

14. A method for identifying a secretory signal sequence on a DNA fragment from a unicellular
 20 microorganism cell host, said method comprising:
 inserting said fragment into a plasmid comprising:
 a replication system functional in an expression cell host;
 a multiple cloning site having at least two restriction sites unique to said plasmid;
 an open reading frame encoding an enzyme, capable of being detected upon secretion, contiguous to said
 25 multiple cloning site and in reading frame with said two unique restriction sites;
 said insertion occurring at at least one of said unique sites to be in reading frame with said open reading
 frame;
 transforming said expression cell host with said insert containing plasmid; and
 growing said transformed cell host in a nutrient medium and detecting secretion by means of said enzyme
 in said medium.

30 15. A method according to Claim 14, wherein said plasmid further comprises a replication system
 functional in a cloning cell host;
 wherein said method further comprises:
 transforming said cloning cell host with said insert containing plasmid to amplify said plasmid; and
 isolating said amplified plasmid and transforming said expression cell host with said amplified plasmid.

35 16. A method according to Claim 14 or 15, wherein said expression cell host is a Bacillus.

17. A method according to any one of Claims 14-16, wherein said unicellular microorganism cell host is
 a Bacillus.

40 18. A method according to any one of Claims 14-17, wherein said enzyme is alpha-amylase, beta-
 lactamase or penicillinase.

19. A method according to any one of Claims 14-18, wherein said plasmid further comprises a
 transcriptional initiation region, where the direction of transcription is from said initiation region through said
 multiple cloning site to said open reading frame and which initiation region is functional at least in said
 expression cell host.

45 20. A method according to Claim 19, wherein said transcriptional initiation region is a Bacillus SPO₂
 transcriptional initiation region.

21. A method for screening a fragment from a unicellular microorganism cell host for a secretory signal
 sequence, said method comprising:

inserting said fragment into at least one plasmid comprising:

50 a replication system functional in an expression cell host;
 a multiple cloning site having at least two restriction sites unique to said plasmid;
 an open reading frame encoding an enzyme, capable of being detected upon secretion, contiguous to said
 multiple cloning site and in reading frame with said two unique restriction sites;
 said insertion occurring at at least one of said unique sites to be in reading frame with said open reading
 frame;

55 wherein when more than one plasmid is employed they differ by at least having different open reading
 frames encoding different enzymes;

transforming said expression cell host with said insert containing plasmid; and
growing said transformed cell host in a nutrient medium and detecting secretion by means of said enzyme
in said medium.

22. A method according to Claim 21, wherein said expression host is a Bacillus.

23. A method according to Claim 21 or 22, wherein said plasmid further comprises a replication system
functional in a cloning cell host;

wherein said method further comprises:

transforming said cloning cell host with said insert containing plasmid to amplify said plasmid; and
isolating said amplified plasmid and transforming said expression cell host with said amplified plasmid.

24. A method according to Claim 23, wherein said cloning cell host is E. coli.

25. In a method for producing a peptide product by recombinant techniques, where a unicellular
microorganism host is transformed with an expression cassette comprising a gene encoding said peptide,
and growing said host under nutrient conditions, whereby said peptide is secreted, the improvement which
comprises:

employing as said gene an open reading frame encoding said peptide joined at its 5' terminus to a
secretory signal sequence according to any one of Claims 9, 10, 12 and 13, wherein said open reading
frame is other than the native open reading frame of said secretory signal sequence.

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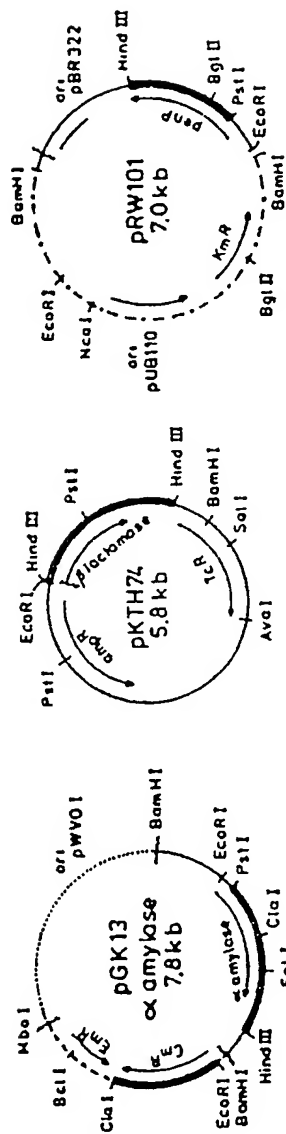
EUROPEAN SEARCH REPORT

Application number

EP · 87 20 0813

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	NUCLEIC ACIDS RESEARCH, vol. 12, no. 13, July 1984, pages 5307-5319, IRL Press Ltd, Oxford, GB; K. OHMURA et al.: "Length and structural effect of signal peptides derived from Bacillus subtilis alpha-amylase on secretion of Escherichia coli beta-lactamase in B. subtilis cells" * Whole article *	1-7, 9, 10, 14-19, 21-24	C 12 N 15/00
X	NUCLEIC ACIDS RESEARCH, vol. 11, no. 6, 25th March 1983, pages 1645-1655, IRL Press Ltd, Oxford, GB; L. DENTE et al.: "pEMBL: a new family of single stranded plasmids" * Figure 1 *	1, 3-7, 9, 10	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
X, P	WO-A-8 503 945 (UNILEVER N.V.) * Pages 36, 37, 64 *	1-5, 7-10	C 12 N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 17-08-1987	Examiner CUPIDO M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

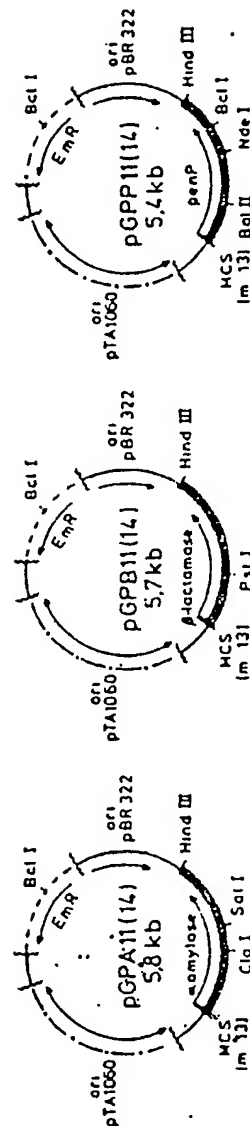
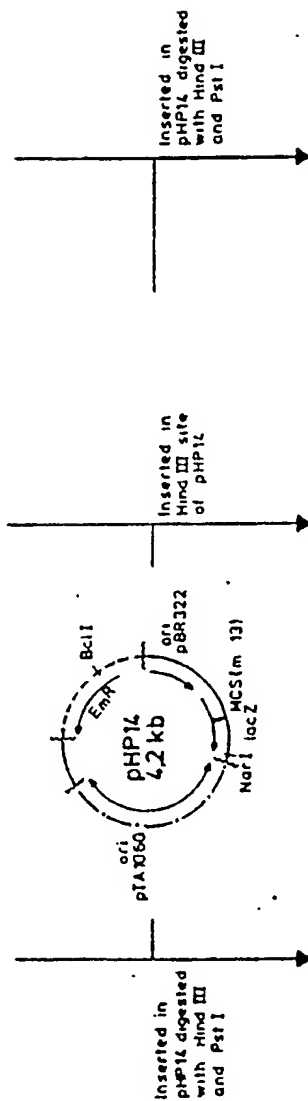
FIG. 1



1.8 kb PstI-Hind III fragment carrying the α amylase gene lacking most of its signal sequence

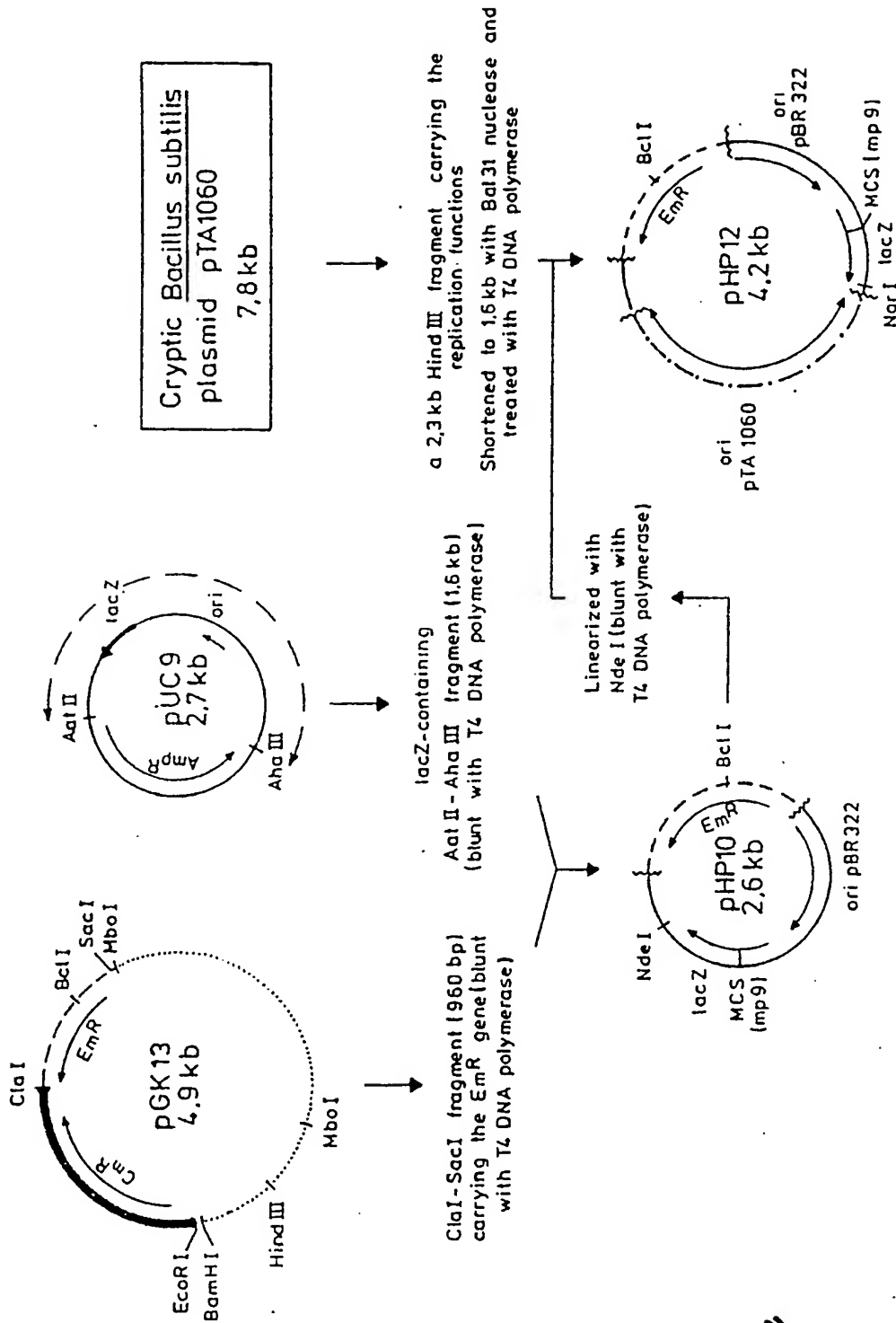
1.5 kb Hind III fragment carrying the β lactamase gene lacking its signal sequence

1.2 kb PstI-Hind III fragment carrying the penP gene lacking part of its signal sequence



Key: — pBR322 — cloned fragments (α amylase, β lactamase, penP)
 - - - pE194 - - - fusion points
 pWV01
 pUB110

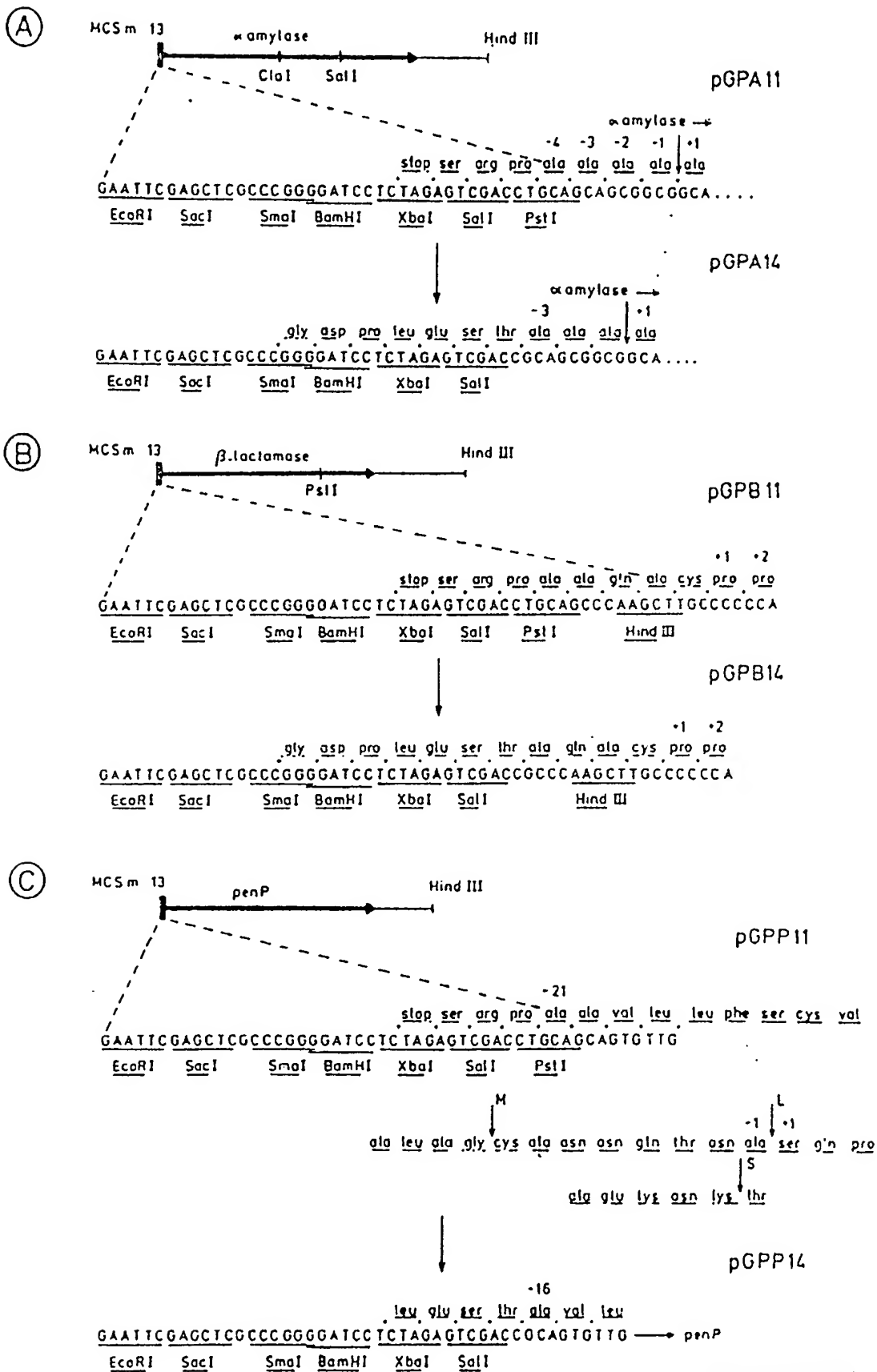
FIG. 2



BAD ORIGINAL

Key: **pC194** **pWV01** — — — **pTA1060**
pE194 — — — **pBR322** ~~~~~ **fusion points**

FIG. 3



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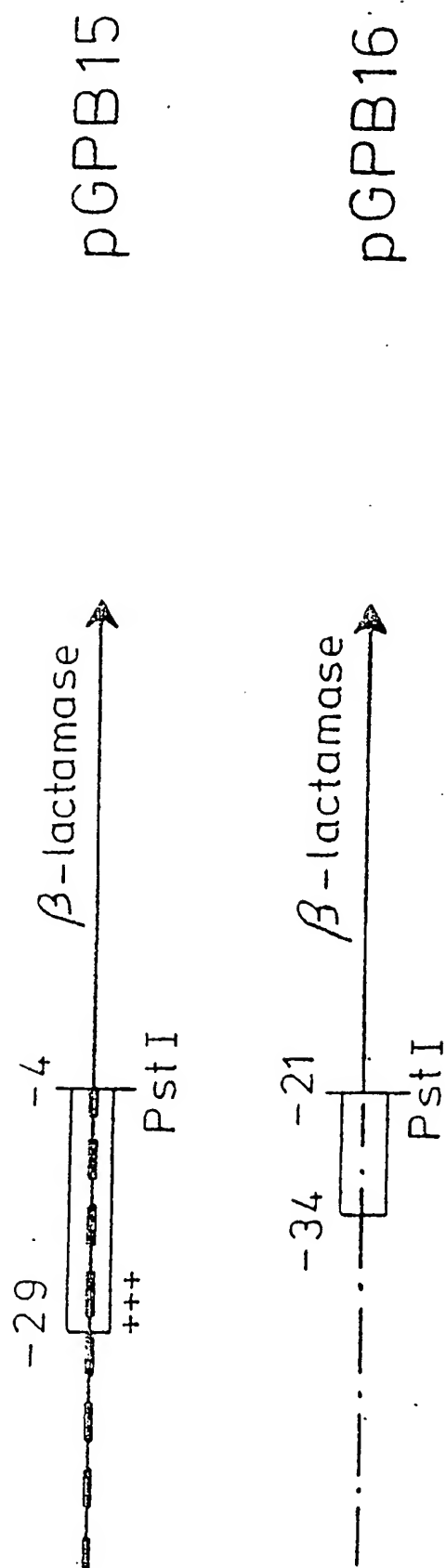


FIG. 4

FIG. 5

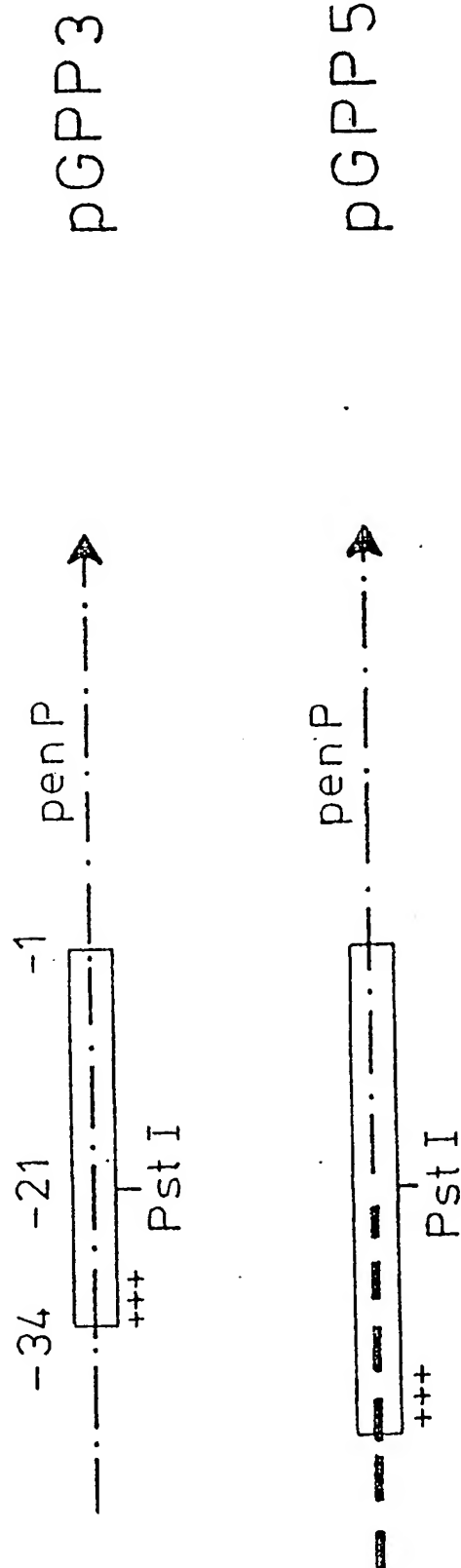


FIG. 6

